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Identification of the adsorbing site of lysozyme onto the hydroxyapatite surface using hydrogen exchange and ^{1}H NMR

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The lysozyme–hydroxyapatite interaction was studied by measuring individual hydrogen–deuterium (H-D) exchange rates of amide protons. The H-D exchange reaction was initiated by transferring the lysozyme adsorbed on hydroxyapatite powder from H_2O into D_2O. After various H-D exchange time periods (pH 7.0, 25°C), the complex was dissociated and the remaining hydrogen label was determined by 2D NMR analysis. The H-D exchange rate of amide protons of residues 9, 11, 13, and 83 was slowed in the hydroxyapatite–lysozyme complex compared with free lysozyme. Residues 9, 11 and 13 positioned at the back of the active site would be the location of the binding site.

Hydrogen exchange; NMR; Hydroxyapatite; Lysozyme; Adsorption

1. INTRODUCTION

The protein–hydroxyapatite interaction has to be understood to elucidate roles of protein in biomineralization and to understand mechanisms related to protein chromatography on a hydroxyapatite column. There has been no method available to measure binding sites and conformation of the protein adsorbed on the solid surface. Recent progress in NMR spectroscopy has made it feasible to acquire complete proton resonance assignments for small proteins, and their structure in solution was determined. However, high resolution NMR is at present not applicable for proteins adsorbed on a solid surface, because of low resolution and sensitivity. In the present experiment, hen egg white lysozyme was used as an adsorbate because of its complete proton resonance assignments [1] and its well known behavior on hydroxyapatite chromatography [2]. Many derivatives can be used to clarify the roles of functional groups of lysozyme [3]. The method we report here combines protection of the lysozyme by hydroxyapatite against hydrogen exchange labeling and 2D proton NMR. With this method, a protein can recall its conformation and the site of where it had bound to the solid surface.

2. MATERIALS AND METHODS

Hen egg white lysozyme (E.C. 3.2.1.17) was obtained from Sigma and was dialyzed extensively before use. Hyroxyapatite was obtained from Bio-Rad and its specific surface area was 60 m^2/g (BET method).

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Other reagents were of analytical grade. The procedure used for hydrogen exchange on the hydroxyapatite-bound lysozyme was as follows: lysozyme solution in 1 mM phosphate buffer was charged on a hydroxyapatite column (1 g hydroxyapatite, 1.0 cm × 4.5 cm) and the column was washed with the buffer to remove unbound lysozyme. To initiate exchange, the column was washed with D_2O (1 mM phosphate, pD 7.0) and incubated at 25°C for time intervals ranging from 30 min to 7 days in a series of H-D exchange experiments. After various exchange time periods, lysozyme was eluted and the H-D exchange reaction was slowed down with deuteroacetate buffer (100 mM, pD 3.8). After elution, the pD of the solution was readjusted to pD 3.8 with conc. DCl, then it was concentrated to about 3 mM on an Amicon filter (YM 10) for 2D proton NMR observation. For H-D exchange experiments of free lysozyme, freeze-dried lysozyme was dissolved into D_2O (1 mM phosphate, pD 7.0) and the exchange reaction was initiated. After incubation at 25°C for the same time intervals with the bound experiments, pD of the solution was adjusted to 3.8 with 100 mM DCl and the remaining hydrogen label on individual amide sites was determined by 2D NMR analysis. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker AM400 spectrometer equipped with an Aspect-3000 computer. The phase-sensitive DQF-COSY spectra [4] were recorded with suppression of the water signal. A spectral width of 6014 Hz was used in both dimensions; 24 transients of 2048 complex data points were recorded for each of 512 t_1 increments. The digital resolution was 4.8 Hz per point in both the f_1 and the f_2 dimensions.

3. RESULTS

Although the overlap of amide proton resonances is substantial in one-dimensional ^{1}H NMR spectra and hampers the observation of the individual amide-proton signals, well resolved cross-peaks could be identified for virtually every amide proton in COSY spectra. The fractional exchange of individual hydrogens under free and bound conditions was then determined from the volume integral of the cross-peaks. The exchange rates could, in principle, be measured for the large majority

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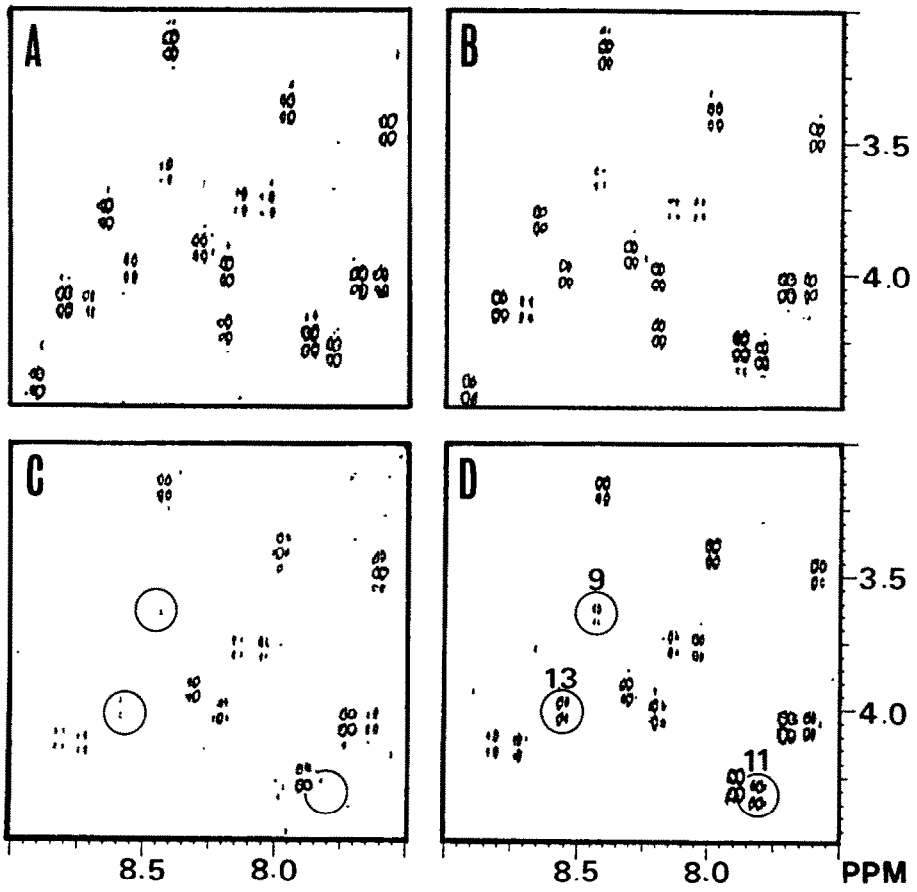

Fig. 1. Part of the ^{1}H NMR COSY spectrum of lysozyme recorded at pH 3.8 and 35°C after partial H-D exchange for 30 min (A) in the absence and (B) in the presence of hydroxyapatite. After incubation for 24 h, some cross peaks (C) disappeared in the absence of hydroxyapatite, however, (D) remained in the presence of hydroxyapatite.

of amide hydrogens with assigned resonances, using this method [5,6]. The COSY spectrum of free lysozyme incubated for 30 min reveals 41 NH-C_αH cross peaks with sufficient resolution, intensity, and H-exchange lifetime to support accurate H-exchange measurements (Fig. 1). After one week of exchange in the free state, 13 of these NH groups remained unexchanged and could not serve as probes for further slowing of the H exchange in the complex at the pH and temperature used in these studies (pH 7.0 and 25°C). Fig. 1 shows the sections of 2D NMR spectra of representative lysozyme samples after partial H-D exchange in bound and free states. It is evident that the amides of Ala^9, Ala^{11} and Lys^{13} exchange more slowly in the bound state. Significant differences were also observed for Leu^{83}; they are the most prominent differences between the two states and are all slower in the bound than in the free state. The H-D exchange rates for the 28 amide protons that did show significant exchange in this time period were obtained by plotting the decreasing intensity of each NH-C_αH cross peak against time of exchange in D_2O. Exchange rates in the free lysozyme (k_{free}) and in the bound lysozyme (k_{bound}) are listed in Table I.

4. DISCUSSION

Hydroxyapatite–protein interactions have been extensively investigated in order to understand the adsorption–elution process in hydroxyapatite chromatography [2]. It has been proposed that lysozyme is a basic protein and adsorbed onto P crystal sites on the surface

Table I

Effects on lysozyme H-D exchange due to adsorption onto hydroxyapatite

Residue	k_{free} (h^{-1})	k_{bound} (h^{-1})	k_{free}/k_{bound}
9	0.19	0.038	5.0
11	0.15	0.031	4.8
13	0.08	0.006	13
83	0.016	0.004	4.0

Residues with protection factors greater than 2 are listed.

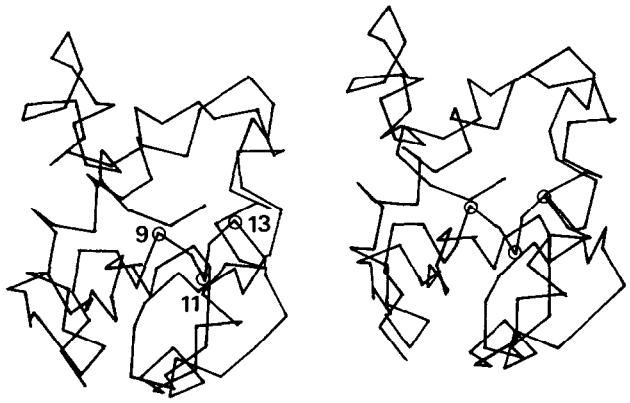

Fig. 2. Stereo view of the lysozyme molecule. Circles indicate residues whose amide protons are retarded in H-D exchange reaction by adsorption on hydroxyapatite.

of hydroxyapatite through basic functional groups and that elution of the molecule is carried out by competition with cations of the buffer. However, little is known of the conformation and the adsorption sites of lysozyme onto hydroxyapatite. The results in Table I show that a majority of the exchangeable hydrogens were little affected in the hydroxyapatite complex, thereby indicating that the overall structure of lysozyme and the stability of regions outside the binding site were little perturbed by the interaction with hydroxyapatite. In contrast, 4 residues, Ala^{9}, Ala^{11}, Lys^{13}, and Leu^{83} underwent a 4- to 10-fold reduction in H-exchange rate on binding to the crystal surface. These values of protection factors, k_{free}/k_{bound}, are smaller than those of cytochrome *c* bound to antibody [7], probably because both the association constant, K_a, and the contact surface area of lysozyme–hydroxyapatite are smaller than that of the antigen–antibody complex. As illustrated in Fig. 2, these slowed NH protons are located at the back surface of the active site of lysozyme molecule. For residue 83 it remains to be determined whether retardation of the exchange rate is due to the remote slowing effect by adsorbing site around Lys^{13} or to another species of lysozyme bound around Leu^{83}. Kawasaki [8] proposed a lysozyme binding model on the basis of the theory of hydroxyapatite chromatography. He claimed that several positive charges clustering at the lysozyme surface bind to the hydroxyapatite surface. Around the protected residues against exchange, there are 4 basic residues, Lys^{13}, Arg^{128}, Arg^{14} and Lys^{1}, all or some of which can be candidates as a binding site. This part of the lysozyme is at the back of the active site, therefore, the lysozyme bound to the hydroxyapatite may have enzyme activity. It has been reported that lysozyme adsorbed onto hydroxyapatite is enzymatically active [9].

The unique method described here makes it feasible to measure the adsorbing sites and conformation of protein adsorbed onto a solid surface.

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REFERENCES

[1] Redfield, C. and Dobson, C.M. (1988) Biochemistry 27, 122–136.
[2] Gorbunoff, M.J. (1985) Methods Enzymol. 182, 329–339.
[3] Ueda, T., Yamada, H., Sakamoto, N., Abe, Y., Kawano, K., Terada, Y. and Imoto, T. (1991) J. Biochem. 110, 719–759.
[4] Rance, M., Sorensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 117, 479–485.
[5] Richarz, R., Sehr, P., Wagner, G. and Wüthrich, K. (1979) J. Mol. Biol. 130, 19–30.
[6] Wagner, G. and Wüthrich, K. (1982) J. Mol. Biol. 160, 343–361.
[7] Paterson, Y., Englander, S.W. and Roder, H. (1990) Science 249, 755–759.
[8] Kawasaki, K. (1978) J. Chromatogr. 157, 7–42.
[9] Schilling, K.M. and Bowen, W.H. (1988) J. Dent. Res. 67, 2–8.